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Differential expression of nitrate reductase in response to potassium and sodium nitrate: real-time PCR analysis

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Abstract

Cucurbita pepo L. plants were grown in plastic pots with 2 kg washed sand per pot at three levels of potassium and sodium nitrate supplies to investigate the effects of nitrate supply on nitrate reductase activity and nitrate reductase expression in the leaves of the plants. Results demonstrated that starvation leads to decrease nitrate reductase activity and nitrate reductase expression. Supplementation of sodium and potassium nitrate had stimulatory effects on nitrate reductase activity and nitrate reductase expression in a dose dependent manner. However, potassium nitrate supplementation increased nitrate reductase activity and nitrate reductase expression more than sodium nitrate supplementation. In conclusion, both sodium and potassium nitrate, as inducers, had significant effects on both nitrate reductase activity and nitrate reductase expression in low concentrations. At high nitrate supplies, nitrate reductase activity and nitrate reductase expressions were suppressed probably due to nitrogen metabolite feedback inhibition and toxicity.

Keywords: nitrate salts supply, nitrate reductase activity, nitrate accumulation, nitrite production, feedback inhibition.

Introduction

Nitrate is a major source of inorganic nitrogen utilized by most plants. Nitrate in soil after being taken up by plant roots is 1) reduced in root cells 2) stored in the root cells vacuoles 3) transported in the xylem through transpiration stream to be reduced in the leaves or 4) stored in the leaf vacuole cells to be released later and reduced in the cytosol (Miller and Cramer, 2004; Jackson et al., 2008). The nitrate assimilatory pathway is mediated by two enzymes, nitrate reductase (EC 1.6.6.2) and nitrite reductase (EC 1.7.7.1), which catalyze the stepwise reduction of nitrate to nitrite and nitrite to ammonia, respectively. Ammonia itself can enter amino acids, proteins and nucleic acids using carbon skeletons derived from metabolites, synthesized by photosynthesis. Nitrate is reduced more efficiently in the leaves because of the readily available reductants, energy supply and carbon skeletons provided by photosynthesis. Nitrate is first reduced to nitrite in the cytosol by nitrate reductase. Nitrite is then translocated to the chloroplasts where it is reduced to ammonium by nitrite reductase (Oaks, 1992 &1994). Most steps in the nitrate assimilatory pathway are nitrate inducible. Nitrate strongly stimulates the transcription of nitrate transporters, nitrate reductase and nitrite reductase genes (Chen et al., 2004; Skrdleta et al., 1979; Stohr, 1999; Sivasankar et al., 1997). The molecular mechanism for the induction of this pathway by nitrate supply is not clearly known. Thus, in this study to gain more insight of this mechanism we examined the effects of potassium and sodium nitrate supplies on nitrate reductase activity and nitrate reductase expression in the leaves of *Cucurbita pepo* L. as a model.

Results

Effects of nitrate supply on the nitrate accumulation in the leaves

The concentration of nitrate in the leaves (nitrate accumulation) of starved plants was taken as 100%. In plants supplied with potassium nitrate at 25, 50, and 100mM, the nitrate concentrations were 388%, 621%, and 510%, respectively. Corresponding values in plants supplied with sodium nitrate at 25, 50, and 100mM were 286.5%, 552%, and 461.5%, respectively. Thus, both supplementation of sodium and potassium nitrate increased nitrate accumulation in a dose dependent manner. However, in plants supplemented with potassium nitrate the concentrations of nitrate in the leaves were higher than in plants supplemented with sodium nitrate ($F_{7,16}$ value = 151.37, p = .001, n = 3) (Figure 1).

Effects of nitrate supply on the nitrite production in the leaves

The concentration of nitrite in the leaves of starved plants was taken as 100%. In plants supplied with potassium nitrate at 25, 50, and 100mM, the nitrite concentrations were 160.2%, 181.5%, and 150.4% of control plants respectively. However, in plants supplied with sodium nitrate at 25, 50, and 100mM the corresponding vales were 137.8%, 157.2%, and 129.6%, respectively.

Thus, both sodium and potassium nitrate supplementation increased nitrite accumulation in a dose dependent manner. However, in plants supplemented with potassium nitrate the concentrations of nitrite in the leaves were higher than in plants supplemented with sodium nitrate ($F_{7,16}$ value = 49, p = .001, n = 3)(Figure 2).

Effects of nitrate supply on the nitrate reductase activity in the leaves

The activity of nitrate reductase in the leaves of starved plants was taken as 100%. In plants supplied with potassium nitrate at 25, 50, and 100mM the enzyme activity increased by 170%, 213%, and 123%, respectively. However, in plants supplied with sodium nitrate at 25, 50, and 100mM the enzyme activity increased by 147%, 163%, and 119%, respectively. Thus, both sodium and potassium nitrate supplementation increased nitrate reductase activity in a dose dependent manner. However, in plants supplemented with potassium nitrate the activity of nitrate reductase in the leaves was higher than in plants supplemented with sodium nitrate ($F_{7,16}$ value = 53.81, p = .001, n = 3) (Figure 3).

Effects of nitrate supply on the nitrate reductase specific activity in the leaves

The specific activity of nitrate reductase in the leaves of starved plants was taken as 100%. In plants supplied with potassium nitrate at 25, 50, and 100mM the enzyme activity increased by 154%, 184%, and 115%, respectively. But in plants supplied with sodium nitrate at 25, 50, and 100mM the enzyme activity increased by 133%, 139%, and 105%, respectively. Thus, both sodium and potassium nitrate supplementation increased nitrate reductase specific activity in a dose dependent manner. However, in plants supplemented with potassium nitrate the specific activity of nitrate reductase in the leaves was higher than in plants supplemented with sodium nitrate ($F_{7,16}$ value = 19.146, p = .001, n = 3) (Figure 4).

Effects of nitrate supply on the nitrate reductase expression in the leaves

In starved plants the mRNA expression was taken as 100%. In plants treated with the 25, 50, and 100 mM potassium nitrate, the expression increased by 360%, 440%, and 270 %, respectively. But in plants supplied with sodium nitrate at 25, 50, and 100mM concentrations, the values were 160%, 420%, and 110%, respectively. Thus, both sodium and potassium nitrate supplementation increased the level of nitrate reductase mRNA in a dose dependent manner. However, in plants supplemented with potassium nitrate, the mRNA of nitrate reductase in the leaves was higher than in plants supplemented with sodium nitrate ($F_{7,16}$ value = 36.53, p = .001, n = 3) (Figure 5).

Discussion

Our results indicate that both sodium and potassium nitrate supplementation increase nitrate accumulation in a dose dependent manner and in plants supplemented with potassium nitrate the accumulation of nitrate in the leaves was higher than in plants supplemented with sodium nitrate (Figure1). Nitrate in soil is absorbed by root cells mediated by nitrate transporter. Nitrate is mainly reduced in the roots or stored in root cells vacuoles.

However, the excess nitrate is transported to the leaves where it is reduced in the leaf cell cytoplasm and the excess of which is stored in leaves cells vacuoles. Thus nitrate accumulation in the leaves increased with nitrate supply. Nitrate uptake and accumulation is highly related to endogenous nitrate. The efficiency of net nitrate uptake rate is under negative feedback control by nitrate accumulation (Skrdleta et al., 1979; Chen et al., 2004; Orsel et al., 2002). Therefore, when nitrate supply is higher than the plant demand, the decrease in nitrate accumulation might be due to the decrease of nitrate uptake as a result of the negative feedback regulation by accumulated nitrate. Thus an excess of nitrate supply in soil will lead to a decrease in nitrate uptake and accumulation, which will result in nitrate build up in soil, and nitrate pollution (Stohr, 1999; Sivasankar et al., 1997). The rate of nitrate uptake relies on the activity of nitrate transport systems in the plasma membrane of root cells. External factors, such as nitrate concentration, light intensity, and temperature, as well as internal factors such as nitrogen metabolites (ammonium and glutamine) all regulate the rate of nitrate uptake. Two classes of proteins encoded by the NRT2 and NRT1 (nitrate transporter1 and 2) gene families (Miller et al., 2007; Orsel et al., 2002) are believed to regulate nitrate acquisition at the level of transcription. Exposure of roots to nitrate causes the induction of NRT2 transcripts, which leads to nitrate uptake by positive feed forward, whereas metabolites resulting from nitrate reduction, most likely ammonia and glutamine, down regulate NRT2 (Remans et al., 2006). Glutamine represses the abundances of the putative NRT2 transcripts which will result in less nitrate uptake. In addition, NRT2 is post-translationally phosphorylated and deactivated by phosphoprotein binding proteins (Walch-Liu and Forde, 2008; Remans et al. 2006).

In addition, our results indicate that both sodium and potassium nitrate supplementation increase nitrite accumulation in a dose dependent manner and in plants supplemented with potassium nitrate the concentrations of nitrite in the leaves were higher than in plants supplemented with sodium nitrate (Figure 2). Nitrate can be reduced directly by nitrate reductase. The nitrate reductase activity increased rapidly with the increase in internal nitrate within the lower nitrate concentration ranges, and started to decreased when endogenous nitrate was high (Vidal et al., 2010; Debouba et al., 2006). These results suggest that nitrate has a positive effect on nitrate reductase activity when its supply is low (Figure 3, 4). Nitrate reductase activity is considered as a limiting factor for nitrate assimilation in higher plants, and is induced by nitrate. In our experiments, only at the lower nitrate supplies, the nitrate had a positive effect on nitrate reductase activity, while at the higher nitrate supplies; the nitrate reductase activity decreased considerably (Figure 3, 4). The present results suggest that the relationship between nitrate reductase activity and nitrite accumulation is dependent on the exogenous nitrate. At the molecular level, nitrate reductase can be phosphorylated at a serine residue, creating a binding site for 14-3-3 proteins, and binding of 14-3-3 protein inhibitors to phosphorylated nitrate reductase inactivates this enzyme completely in the presence of divalent cations (Huber et al., 1996; Huber et al., 2002; Huber, 2007; Lambeck et al., 2010). In addition, our results indicate that both sodium and potassium nitrate supplementations increase nitrate reductase expression in a dose dependent manner. However, in plants supplemented with potassium nitrate the expression of nitrate reductase in the leaves were higher than in plants supplemented with sodium nitrate (Figure 5). Nitrate at low concentrations

strongly stimulates the transcription of nitrate reductase activity gene, which leads to enhanced nitrite production (Faure et al., 1991). One of the major findings of our studies is the different effects of potassium and sodium nitrate on the nitrate assimilation pathway. In all cases studied, potassium nitrate was more effective than sodium nitrate. As an essential macronutrient for higher plants, potassium has several functions such enzymes activation (more than 50 enzymes), neutralization of organic and inorganic anions, maintenance of cytosolic pH, cell turgidity and phloem transport. Thus, the rates of some reactions are controlled by the rate at which K enters the cell (Winter and Huber, 2000). The activation of some carbon reduction cycle enzymes by K and its involvement in ATP production is important in regulating the rate of photosynthesis. The ATP is used as the energy source for many other chemical reactions. The electrical charge balance at the site of ATP production is maintained with K ions. When plants are K deficient, the rate of photosynthesis and the rate of ATP production are reduced, and all of the processes dependent on photosynthetic ATP production are slowed down (Page and Cera, 2006). On the other hand, sugars produced in photosynthesis must be transported through the phloem to other parts of the plant for utilization and storage. Plants transport systems use energy in the form of ATP. If K is inadequate, less ATP will be available, and the transport system will be affected. An adequate supply of K helps to keep all of these processes and transportation systems function normally (Karley and White, 2009; Mathis, 2009). Potassium also plays a major role in the transport of solutes throughout the plant in the phloem. A sufficient supply of K is essential for efficient operation of these systems (Karley and White, 2009; Mathis, 2009). Potassium is required for every major step of protein synthesis. The reading of the genetic code in plant cells to produce proteins and enzymes that regulate all growth processes would be impossible without adequate K. When plants are deficient in K, proteins are not synthesized despite an abundance supply of available nitrogen. Instead, protein precursors such as amino acids, amides and nitrate accumulate (Armengaud et al., Y. 2009; Karley and White, 2009). In protein synthesis, K is likely responsible for the elongation of peptide molecules. The enzyme responsible for synthesis of starch (starch synthetase) is activated by K. Thus, with inadequate K, the level of starch declines while both soluble carbohydrates and nitrogen metabolites accumulate. Photosynthetic activity affects the rate of sugar formation for ultimate starch production. Under high K levels, soluble carbohydrates are efficiently moved from sites of production and will be converted to starch in storage organs (Li et al., 2009; Amtmann and Armengaud, 2009; Armengaud et al., 2009; Luan et al., 2009). However, sodium is not an essential element and it cannot be expected to have a specific role in the metabolic activities of plants. In high concentration, sodium can wholly replace potassium. In such circumstances, sodium is ineffective as a substitute for potassium and reduces the positive physiological roles of potassium (Horie and Schroeder, 2004; Hasegawa et al., 2000; Luan et al., 2009).

Materials and methods

Plant materials and treatments

Cucurbita pepo L. plants were grown in standard nutrient solution (KNO_3 5mM, $Ca(NO_3)_2$ 5mM, $MgSO_4$ $7H_2O$ 2mM, KH_2PO_4 1mM, NaFe-EDTA 0.1mM, H_3BO_3 11.5mM, $MnCl_2 \cdot 4H_2O$ 4.6mM, ZnSO4 $7H_2O$ 0.2mM, Na_2MO_4

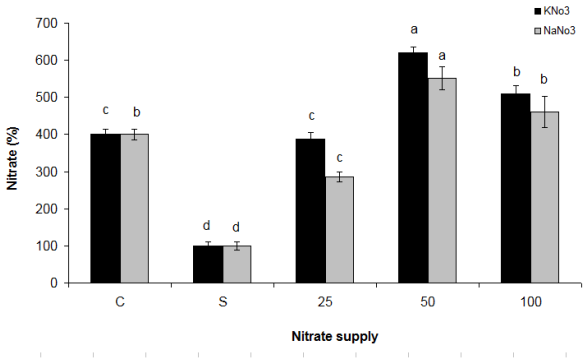

Fig 1. Effects of nitrate concentration on nitrate uptake in starved plants. Plants were grown in standard nutrient solution for 21 days. Plants starved for 1 week, then used for induction experiment with potassium and sodium nitrate (25, 50, 100 mM) for three days. Means with the same letter within an assay are not significantly different as determined by SPSS software version 16 at $p< 0.05$.

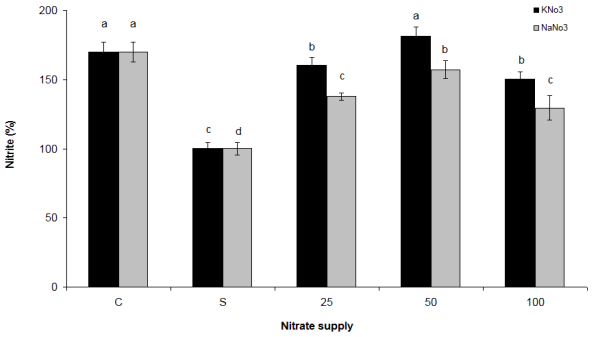

Fig 2. Effects of nitrate concentration on the nitrite concentration in the starved plants. Plants were grown in standard nutrient solution for 21 days. Plants starved for 1 week, then used for induction experiment with potassium and sodium nitrate (25, 50, 100 mM) for three days. Means with the same letter within an assay are not significantly different as determined by SPSS software version 16 at $p< 0.05$.

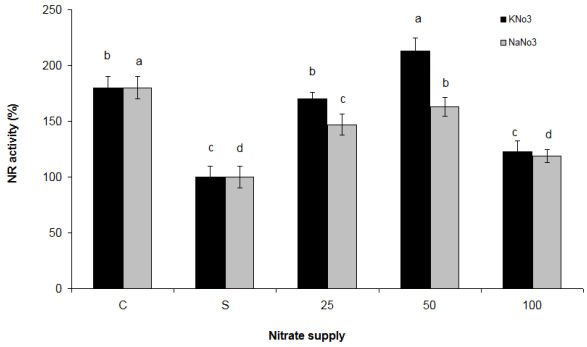

Fig 3. Effects of nitrate concentration on the nitrate reductase activity in the starved plants. Plants were grown in standard nutrient solution for 21 days. Plants starved for 1 week, then used for induction experiment with potassium and sodium nitrate (25, 50, 100 mM) for three days. Means with the same letter within an assay are not significantly different as determined by SPSS software version 16 at $p< 0.05$.

0.12mM and $CuSO_4$ $5H_2O$ 0.08mM) for 3 weeks. Plants were then divided into two groups; 1) grown in standard nutrient solutions (control), and 2) grown in media without nitrate (starved plants) for one week. Starved plants were subdivided into three groups; 1) grown in media without nitrate for four days, 2) grown in various concentrations of potassium nitrate (25, 50 and 100 mM, equivalent to 122, 245, 490 mg KNO_3/ kg sand) for 3 days, 3) grown in various concentrations of sodium nitrate (25, 50 and 100 mM, equivalent to 106, 212, 425 mg $NaNO_3$/kg sand) for 3 days. Twenty four hours after nitrate supplementation, the leaves were harvested and frozen in liquid nitrogen.

Nitrate/nitrite assay

Frozen leaves were powdered with a mortar and pestle, solubilized in phosphate buffered saline pH 7.4 and the extract was centrifuged at 6,000g for 15 min. The supernatant was stored at -70°C. Nitrate was determined by Griess reagent using sodium nitrate as standards (Miranda et al., 2001). Briefly, to 100µl of culture medium, 100µl of vanadium chloride (III) (8mg/ml) was added. After 40 min, 50µl of Griess reagents [1:1 (v/v) of 0.1% naphthylethylenediaminedihydrochloride (NED) in H_2O + 2% sulphanilamide in 5% H_3PO_4] was added and incubated at 37°C for 10 min and the absorbance was read at 540 nm. Results were reported as percentage of starved plants.

Nitrate reductase activity assay

Nitrate reductase assays were performed by reduction of nitrate to nitrite (Brunetti and Hageman, 1979). One unit of enzyme activity is defined as the production of 1 µM nitrite per min. Specific activity described as enzyme activity divided by total protein content and was shown as unit of activity per gram of protein. Results were reported as percentage of starved plants.

RNA extraction and reverse transcription

Total RNA was extracted from plant leaves using RNX-Plus (Cinnagene, Iran) according to the manufacturer's protocol. DNase treatment was carried out using Fermentas DNase Kit according to the manufacturer's instructions., and the purity of RNA was checked after separation of the RNA samples on the agarose gel and visualizing them by ethidium bromide staining and also by OD260/OD280 absorption ratio (>1.8). Two µg of DNase-treated RNA was used for first strand cDNA synthesis, using 100 pmol oligo-dT (18 mer), 15 pmol dNTPs, 20 U RNase Inhibitor and 200 U M-Mulv reverse transcriptase (all from Fermentas) in a 20 µl final volume.

Relative real-time PCR

Real-time PCR for the quantification of nitrate reductase mRNA was conducted using SYBR Premix Ex Taq II reagent (Takara, Japan). cDNAs were amplified by SYBR Premix Ex Taq II in a 20 µl reaction mixture containing 0.4 µl of each gene-specific primer (10 µM), 2 µl cDNA, 10 µl 2x buffer, and 7.2 µl ddH_2O. The specific primer pairs for nitrate reductase were designed as follows: 5'- G A C A T C A T T C T C G C C T A T -3' (forward) and 5'- C A C T T C A C C A T T C T A C C A -3' (reverse). The glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene was used as the reference gene with the primers 5'-G A G G A G T T C G G C A T C G T G A A G GGA-3' (forward) and 5'-T G G G G C A A C A C A A G G G A C A C A G -3' (reverse). The

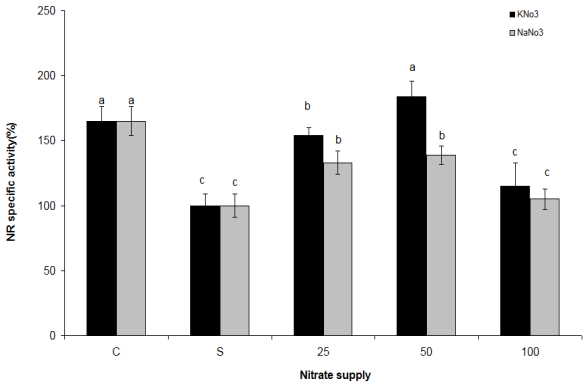

Fig 4. Effects of nitrate concentration on the nitrate reductase specific activity in the starved plants. Plants were grown in standard nutrient solution for 21 days. Plants starved for 1 week, then used for induction experiment with potassium and sodium nitrate (25, 50, 100 mM) for three days. Means with the same letter within an assay are not significantly different as determined by SPSS software version 16 at $p< 0.05$.

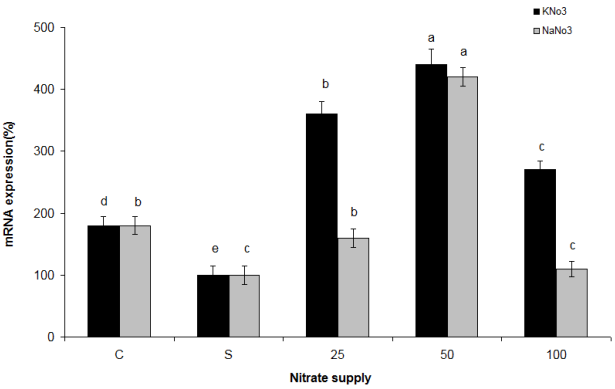

Fig 5. Effects of nitrate concentration on mRNA production in the starved plants. Plants were grown in standard nutrient solution for 21 days. Plants starved for 1 week, then used for induction experiment with potassium and sodium nitrate (25, 50, 100 mM) for three days. Means with the same letter within an assay are not significantly different as determined by SPSS software version 16 at $p< 0.05$.

amplification reactions were carried out in a lineGeneK thermal cycler (Bioer, China) under the following conditions: 2 min at 94 oC, 40 cycles of 94 oC 10 sec, 57 oC 15 sec and 72 oC 30 sec. After 40 cycles, the specificity of the amplifications was tested by heating from 50°C to 95°C, resulting in melting curves. Data were analyzed as described before (Pfaffl, 2001). Results are expressed as percentage of starved plants.

Statistical analysis

Data were analyzed as a completely randomized design with three replications. Data were expressed as means ± standard deviation (SD). Analysis of variance (ANOVA) was employed to investigate significant differences between treatments and then testing for differences between means was by Duncan's new multiple range test and SPSS software version 16 at $p<0.05$.

Conclusion

In conclusion our results indicate that nitrate supply stimulates nitrate uptake, nitrate reduction, nitrate reductase activity, and nitrate reductase expression at low concentrations of potassium and sodium nitrate. But at high nitrate supplies, nitrate metabolites including ammonium and glutamine suppress nitrate uptake, nitrate reduction, nitrate reductase activity, and nitrate reductase expression. In addition, the positive effects of potassium are probably due to its effects on enzyme activity, photophosphorylatoion, sugars transport, water and nutrient transport, protein synthesis, and carbohydrate metabolism. Further experiments are required to definitely demonstrate this hypothesis.

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